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# The Mechanism of Action of Lutropin on Regulator Protein(s) Involved in Leydig-Cell Steroidogenesis

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The dependence on lutropin of the synthesis of a proposed short-half-life protein regulator involved in Leydig-cell steroidogenesis was investigated. This was carried out by determining the effect of the protein-synthesis inhibitor cycloheximide, added before and during incubations with lutropin (and/or dibutyryl cyclic AMP), on the rate of testosterone production in suspensions of purified Leydig cells from adult rat testes. The Leydig cells were preincubated in Eagle's medium for 2.5 h followed by 30 min incubation with and without cycloheximide. The inhibitor was removed by washing the cells and then lutropin was added and testosterone concentrations were determined after incubation of the cells at 32°C. No significant effect of cycloheximide pretreatment on lutropin-stimulated steroidogenesis was found during 60 min incubation. This was in contrast with the complete inhibiting effect of cycloheximide when it was added with the lutropin. The pretreatment experiments with cycloheximide were repeated in the presence of dibutyryl cyclic AMP and elipten phosphate (to inhibit cholesterol side-chain cleavage) followed by incubation with lutropin. After 5, 10, 20 and 60 min of incubation, testosterone concentrations were $61 \pm 3$, $46 \pm 3$, $27 \pm 4$ and $18 \pm 4\%$ lower than in the cells pretreated without cycloheximide respectively (means ± S.E.M., $n = 4$–6). In the cells not pretreated with cycloheximide and in the absence of lutropin, testosterone production increased from $1.36 \pm 0.5$ to $36.5 \pm 1.0$ ng/$10^6$ cells during 20 min of incubation, after which no further increase occurred. Pretreatment of the cells with cycloheximide decreased these testosterone concentrations by 65, 46, 42 and 36% in the 5, 10, 20 and 60 min incubations respectively (mean values, $n = 2$–4). It is apparent from these results that inhibition of steroidogenesis only occurs if protein synthesis is inhibited in the presence of lutropin or cyclic AMP. A new hypothesis is put forward to explain these findings: it is proposed that lutropin affects the stability of a precursor of a regulator protein by converting it from a stable (inactive) to an unstable (active) form with a short half-life.

It has previously been demonstrated that lutropin (LH) stimulation of Leydig-cell steroidogenesis is dependent on protein synthesis (Hall & Eik-Nes, 1962; Cooke *et al.*, 1975; Mendelson *et al.*, 1975) and that a protein(s) with a half-life of 13 min or less is involved (Cooke *et al.*, 1975). Further work showed that lutropin stimulates the synthesis of a protein of mol.wt. 21000 in adult Leydig cells. This 21000-mol.wt. protein is probably not the proposed regulator protein, because the lag period before induction of this protein by lutropin can be demonstrated to be approx. 2 h compared with less than 5 min for stimulation of steroidogenesis, and in addition the half-life of this protein is longer than 30 min (Janszen *et al.*, 1977). Because of the kinetics of lutropin stimulation of testosterone production and the evidence obtained for the lutropin-independent synthesis of RNA molecules involved in steroidogenesis (Cooke *et al.*, 1978), it has been proposed that the RNA(s) and regulator protein(s) required for stimulation of Leydig-cell steroidogenesis are synthesized continuously and independently of lutropin (Cooke *et al.*, 1978). If this hypothesis is true, then it should be possible to inhibit the production of the short-half-life regulator protein(s) in the absence of lutropin, and demonstrate a subsequent lower lutropin stimulation of steroidogenesis. Therefore in the present study the kinetics of testosterone production have been determined for Leydig cells pretreated *in vitro* with cycloheximide followed by removal of the inhibitor before addition of lutropin. Similar experiments have been carried out in which dibutyryl cyclic AMP was

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added with the cycloheximide. The results obtained indicate that lutropin probably does not affect the synthesis of the regulator protein(s) either directly or indirectly, but it does alter the properties of the protein(s), and it is proposed that the mechanism may involve the conversion of a stable long-half-life protein into a short-half-life protein.

## Materials and Methods

Sheep lutropin (NIH-LH-S18; 1.03 i.u./mg) was a gift from the Endocrinology Study Section, National Institutes of Health, Bethesda, MD, U.S.A. [$^3$H]-Leucine (50 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Dibutyryl cyclic AMP and cycloheximide were obtained from Boehringer, Mannheim, Germany. Elipten phosphate (an inhibitor of cholesterol side-chain cleavage) was a gift from CIBA, Basel, Switzerland. Adult male Wistar rats substrain R-Amsterdam, 3–5 months old, were used.

Leydig-cell suspensions from rat testis were prepared and purified by centrifugation through Ficoll and Dextran solutions as described before (Janszen *et al.*, 1976). The Leydig-cell suspensions were incubated in Eagle's (1971) medium (0.1 ml containing $3 \times 10^6$ cells/ml) as indicated in the Schemes given in the Results section. Testosterone was determined in the cells plus incubation medium and the incorporation of [$^3$H]leucine into Leydig-cell proteins was determined as described previously (Verjans *et al.*, 1973; Cooke *et al.*, 1975). When added, 1 mM-dibutyryl cyclic AMP, 350 $\mu$g of elipten phosphate/ml and 2.5 $\mu$g of cycloheximide/ml were used.

## Results

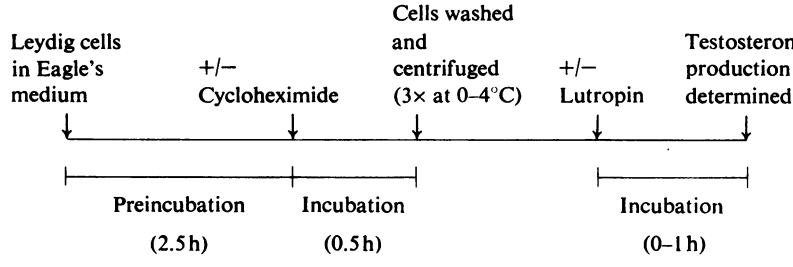
*Effect of cycloheximide on lutropin stimulation of testosterone production*

A typical experiment on the effect of incubating Leydig cells (which had previously been preincubated for 3 h in Eagle's medium) with lutropin with and without the addition of cycloheximide is shown in Fig. 1. Lutropin caused a rapid stimulation of testosterone production, with testosterone concentrations increasing to approx. 180 ng/$10^6$ cells within 60 min of incubation. When cycloheximide was added this stimulation of testosterone production was completely inhibited.

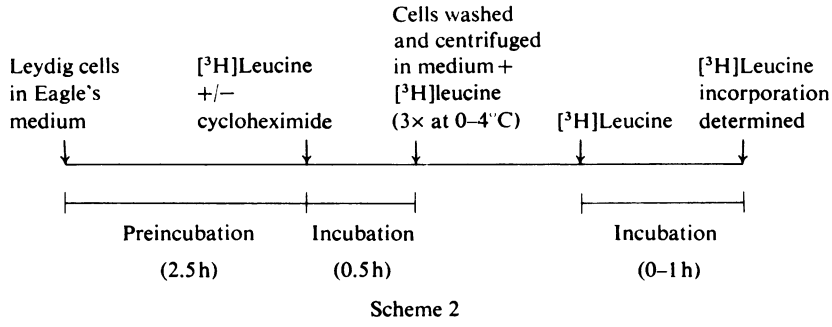
*Effect of pretreating Leydig cells with cycloheximide before addition of lutropin*

To determine if protein synthesis could be fully restored after pretreatment of the cells with cycloheximide, the following experiment was carried out. Leydig cells were incubated with or without cycloheximide (2.5 $\mu$g/ml) for 30 min at 32°C. Duplicate samples of these Leydig-cell suspensions were then either incubated directly with [$^3$H]leucine or washed several times by suspension in Eagle's medium followed by centrifugation at 100*g* for 10 min before incubation with [$^3$H]leucine. The latter incubations were carried out for 60 min at 32°C, and the incorporation of the [$^3$H]leucine into the Leydig-cell protein was determined. The percentage inhibition in the cycloheximide-treated cells compared with the controls was 90±1, 4±5, 0±4 and 4±8% without washing and washing twice, three and four times respectively (means±ranges, $n = 2$). These experiments established that washing the cells two to three times with Eagle's medium is sufficient to remove the cycloheximide from the cells.

Further experiments were then carried out in which the Leydig cells were incubated according to Scheme 1. The resulting testosterone production is given in Fig. 2. Inhibition of protein synthesis before the addition of lutropin had no detectable effect on lutropin-stimulated testosterone production. Both with and without cycloheximide pretreatment of the Leydig cells, lutropin stimulated testosterone production almost linearly during 60 min of incubation with lutropin. In order to eliminate the possibility that this lack of effect of cycloheximide was due to rapid protein synthesis during the washing of the Leydig cells (which was carried out at approx. 5°C), the following experiment was performed. The pretreatment of the cells with and without cycloheximide was carried out as shown in Scheme 2. [$^3$H]Leucine was




Scheme 1




Scheme 2

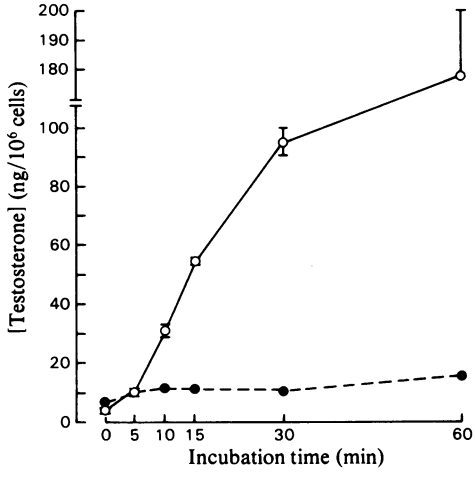



Fig. 1. *Effect of cyclohexamide on lutropin-stimulated testosterone production in Leydig cells*

The Leydig cells were prepared and preincubated as described in the Materials and Methods section for 3h at 32°C. Lutropin (100ng/ml) with (●) and without (○) cycloheximide (2.5 μg/ml) was then added and the cells were incubated at 32°C. Separate incubations were carried out for the times indicated. The total accumulated testosterone concentrations given are means and ranges of duplicate incubations.

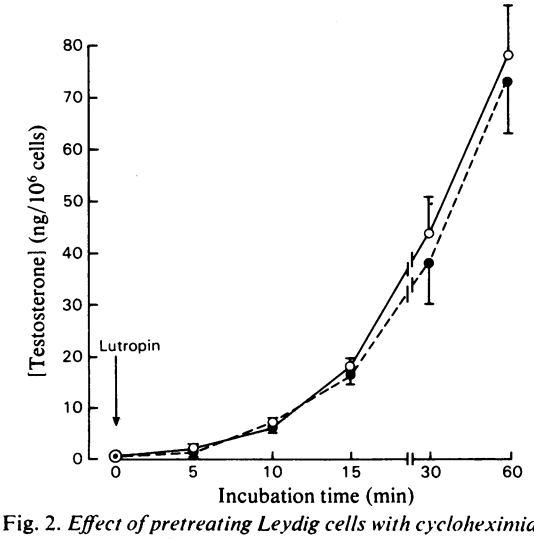


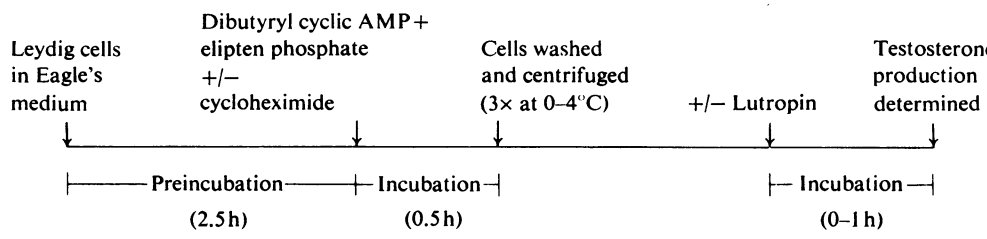

Fig. 2. *Effect of pretreating Leydig cells with cycloheximide on subsequent response to lutropin*

The Leydig cells were prepared as described in the Materials and Methods section and then incubated according to Scheme 1. Separate incubations were carried out for the times indicated. The total accumulated testosterone concentrations given are the means ±S.E.M. for three separate experiments carried out in duplicate. ●, Pretreatment with cycloheximide; ○, pretreatment without cycloheximide.

added to the Eagle's medium used to wash the cells. After the cells had been washed three times, duplicate samples of the cells were removed for determination of the incorporation of [$^3$H]leucine and the remaining cells were incubated for different times at 32°C after addition of further Eagle's medium containing [$^3$H]-leucine. In both the cycloheximide-treated and control Leydig cells there was no detectable incorporation of [$^3$H]leucine into protein during the washing procedure (zero time point in Fig. 3), indicating that no protein synthesis had occurred. This is in contrast with the rapid increase in incorporation of [$^3$H]leucine that occurred at 32°C in both the cycloheximide-treated and control cells (Fig. 3). Similar results were obtained in another experiment (results not shown).

*Effects of pretreating Leydig cells with cycloheximide and dibutyryl cyclic AMP before addition of lutropin*

The results of the experiments described above indicated that cycloheximide only inhibited testosterone production in the presence of lutropin. In order to investigate this possibility further, experiments were carried out according to incubation Scheme 3, in which the cells were stimulated with




Scheme 3

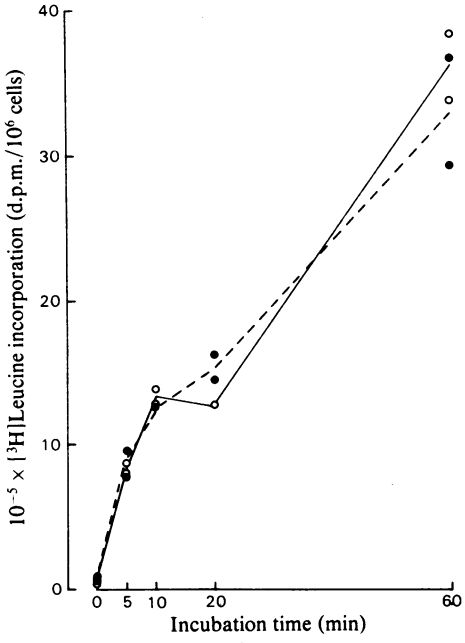



Fig. 3. *Incorporation of [$^3$H]leucine in Leydig cells pretreated with cycloheximide*

The Leydig cells were prepared as described in the Materials and Methods section and then incubated according to Scheme 2. [$^3$H]Leucine was added to the Eagle's medium used to wash the cells and the incorporation of this compound into protein was determined after incubation of the washed Leydig cells for the times indicated. Separate incubations were carried out for each time interval. The results are the means and range of duplicates. ●, Pretreatment with cycloheximide; ○, pretreatment without cycloheximide.

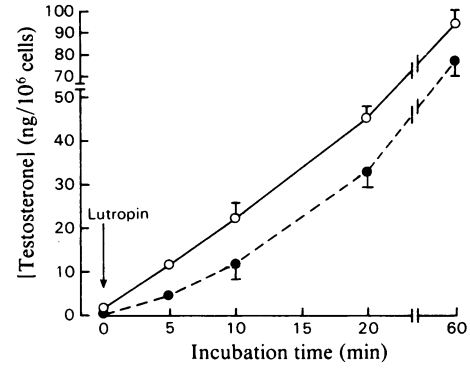



Fig. 4. *Effect of pretreating Leydig cells with cycloheximide, dibutyryl cyclic AMP and elipten phosphate on their subsequent response to lutropin*

The Leydig cells were prepared as described in the Materials and Methods section and incubated according to Scheme 3. Separate incubations were carried out for the times indicated. The total accumulated testosterone concentrations given are the means ± S.E.M. for three separate experiments carried out in duplicate. ●, Pretreatment with cycloheximide etc.; ○, pretreatment without cycloheximide.

dibutyryl cyclic AMP in the presence and absence of cycloheximide. Elipten phosphate, an inhibitor of cholesterol side-chain cleavage, was also added to prevent depletion of cholesterol pools. Dibutyryl cyclic AMP was added in place of lutropin, because, unlike the latter, it can be easily removed from the cells. This was confirmed in preliminary experiments in which the Leydig cells were incubated for 5 min at 32°C with dibutyryl cyclic AMP, followed by washing the cells twice. No stimulation of testosterone production occurred during subsequent incubation of the washed cells at 32°C, thus indicating that this procedure is effective in removing this compound.

The results of pretreating the cells with cycloheximide in the presence of dibutyryl cyclic AMP are shown in Fig. 4. Cycloheximide had a marked inhibitory effect on subsequent stimulation of testosterone synthesis by lutropin especially in the cells incubated for short times. After 5, 10, 20 and 60 min of incubation testosterone concentrations were 61 ± 3, 46 ± 3, 21 ± 4 and 18 ± 4% respectively lower than in the control cells (means ± S.E.M., $n = 6$).

When lutropin was omitted from these control incubations, testosterone production increased from 1.3 ± 0.5 to 36.5 ± 1.0 ng/$10^6$ cells during 20 min of incubation, after which no further increase occurred (Fig. 5). Pretreatment of the cells with cycloheximide

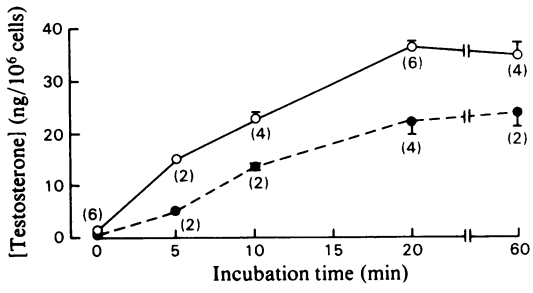



Fig. 5. *Effect of pretreating Leydig cells with cycloheximide, dibutyryl cyclic AMP and elipten phosphate on subsequent steroidogenesis in the absence of lutropin*

The same scheme of incubation was carried out as given in Scheme 3, except that no lutropin was added. Results are means ± S.E.M. ($n$ = 2–6). ●, Pretreatment with cycloheximide etc.; ○, pretreatment without cycloheximide.

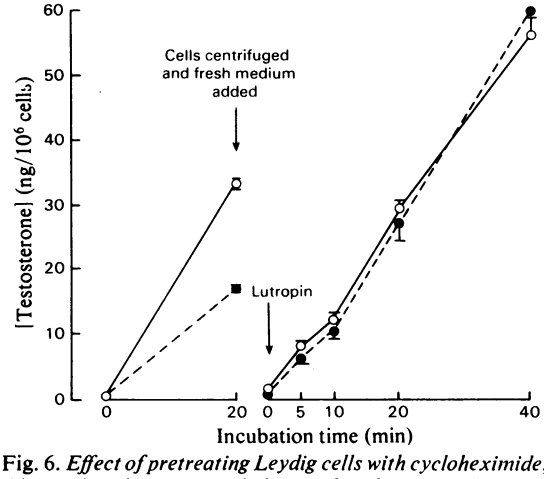



Fig. 6. *Effect of pretreating Leydig cells with cycloheximide, dibutyryl cyclic AMP and elipten phosphate on subsequent steroidogenesis*

The same scheme of incubation was carried out as given in Scheme 3, except that after removal of the cycloheximide etc. the cells were incubated for 20 min in Eagle's medium, then centrifuged and fresh medium containing lutropin was added. ●, Pretreatment with cycloheximide etc.; ○, pretreatment without cycloheximide.

in the presence of dibutyryl cyclic AMP and elipten phosphate decreased these concentrations by 65, 46, 42 and 36% in the 5, 10, 20 and 60 min incubations respectively (mean values, $n$ = 2–4) (Fig. 5).

The last experiment was repeated, except that after removal of the dibutyryl cyclic AMP/elipten phosphate/cycloheximide the cells were incubated for 20 min in Eagle's medium and then centrifuged at 100$g$ for 10 min, resuspended in Eagle's medium containing lutropin and then incubated at 32 C. Fig. 6 shows that during the 20 min incubation the cells pretreated with cycloheximide etc. again gave a lower testosterone production compared with the control. However, no further effect of the pretreatment was obtained after 20 min when lutropin was added.

## Discussion

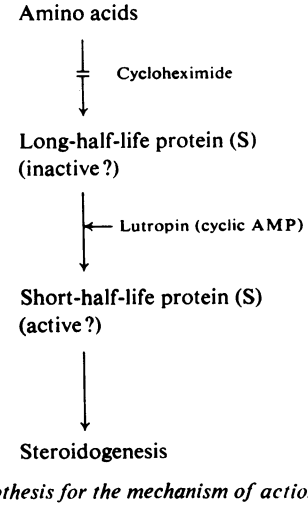
For the three main steroid-producing tissues, the adrenal gland, ovary and testes, it has been proposed that a short-half-life protein or proteins are involved in the tropic-hormone stimulation of steroidogenesis. The evidence for this is based on the rapid inhibitory effect of protein-synthesis inhibitors such as cycloheximide. So far, attempts to demonstrate the presence of this protein(s) have not been successful (see the introduction). It has been suggested for the adrenal gland that because of the very rapid stimulation of corticosteroid biosynthesis (within 24 s), corticotropin does not stimulate biosynthesis *de novo*, but activates a pre-existing protein (Schulster *et al.*, 1974; Lowry & McMartin, 1974). A similar suggestion was made for lutropin stimulation of Leydig-cell steroidogenesis and alternatively that the protein(s) may play a permissive role without any modification by lutropin (Janszen *et al.*, 1977). Further work on Leydig cells has shown that the kinetics of lutropin stimulation of testosterone production are modified by preincubating freshly prepared Leydig cells in Eagle's medium only; the lag time of response to lutropin is decreased from 20–30 min to less than 5 min (Cooke *et al.*, 1977). Furthermore, in contrast with freshly prepared cells, the preincubated cells are stimulated by lutropin independently of RNA synthesis *de novo* (Cooke *et al.*, 1978). This work provided additional evidence that the RNA(s) and proposed regulator protein(s) involved in the stimulation of steroidogenesis are synthesized independently of lutropin.

If the proposed short-half-life protein(s) is synthesized independently of lutropin, then it would be expected that inhibition of protein synthesis in the absence of lutropin, as carried out in the present study, would lead to a rapid depletion of the amount of this protein(s) present in the Leydig cells. However, this was shown not to be the case; no difference in the rate of testosterone production was detected in the cycloheximide-treated cells compared with the controls when lutropin was added after removal of the inhibitor. Thus it would seem that cycloheximide only affects stimulation of steroidogenesis in the presence of lutropin, i.e. that some modification of newly synthesized protein(s) by lutropin is taking place. This hypothesis is strengthened by the results of the

experiments in which the cells were pretreated with cycloheximide in the presence of dibutyryl cyclic AMP; the initial rate of subsequent steroidogenesis was markedly inhibited after removal of the cycloheximide and dibutyryl cyclic AMP. The extent of this inhibition rapidly decreased with time.

It is of interest that in the absence of lutropin the dibutyryl cyclic AMP-treated cells rapidly produced testosterone, which stopped after 20min incubation. This was not due to residual dibutyryl cyclic AMP, hence it could possibly reflect the half-life of an activated protein.

There are two possible explanations of the results presented in this paper. One is to go back to the original hypothesis proposed by Garren *et al.* (1965) for the adrenal cell, i.e. that lutropin stimulated the synthesis of the proposed short-half-life regulator protein(s). The evidence against this is based on: (*a*) the failure to demonstrate the synthesis of this protein; (*b*) the very rapid kinetics of tropic-hormone stimulation of steroidogenesis (induction of new protein synthesis is generally accepted to require hours and not to be possible within minutes or seconds); (*c*) the apparent tropic-hormone independence of RNA synthesis involved in steroidogensis (Schulster, 1974; Cooke *et al.*, 1978). However, as demonstrated in the present study, the effect of cycloheximide does require the presence of lutropin (or cyclic AMP) and it may be concluded that lutropin does influence directly or indirectly the regulator protein(s). The following hypothesis is therefore proposed to explain these results (see Scheme 4). In the absence of lutropin the regulator protein(s) is present in a stable form with a long half-life. Addition of cycloheximide will prevent its further synthesis, but, because of its long half-life, there will be a sufficient pool of this protein(s) for subsequent stimulation of steroidogenesis when lutropin is added. In the presence of lutropin the stable protein is converted to an unstable form with a shorter half-life. Thus in the presence of cycloheximide further synthesis of this short-half-life protein will be inhibited, the pool will be depleted and steroidogenesis will cease. An additional effect of this transformation of a stable protein to an unstable protein is that this process may also be an activation step, which is similar to the suggestion previously made (Lowry & McMartin, 1974; Schulster *et al.*, 1974; Janszen *et al.*, 1977). It remains to be investigated which mechanisms are involved, but they could include direct effects of lutropin on the protein or indirect effects by activation of proteolytic enzymes and/or phosphorylation.




Scheme 4. *Hypothesis for the mechanism of action of lutropin*

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